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(54) Title: METHOD OF INHIBITING NITRIC OXIDE FORMATION

(57) Abstract

A method is disclosed for inhibiting nitric oxide formation in a warm blooded mammal afflicted with an acute or chronic inflammatory disease which comprises administering to said mammal an effective nitric oxide inhibitory amount of methyl-, or 1,1-dimethyl-, or amino-substituted guanidines.

METHOD OF INHIBITING NITRIC OXIDE FORMATION

CROSS-REFERENCE TO RELATED APPLICATIONS

5   This is a continuation-in-part of application Serial No. 08/328,925 filed October 25, 1994, which is in turn, a continuation-in-part of application Serial No. 08/110,915, filed August 24, 1993 a continuation-in-part of application Serial No. 07/843,387, filed February 28, 1992 and application Serial No. 07/906,632, filed June 30, 1992, each of which are a continuation-in-part of application Serial No.
10   07/807,912, filed December 16, 1991.

BACKGROUND OF THE INVENTION

This invention relates to a method of-inhibiting nitric oxide formation in warm blooded mammals and, more particularly, to the administration of methyl-, dimethyl-, or amino-substituted guanidines as inhibitors of nitric oxide production
15   in a host afflicted with acute or chronic inflammatory disease.

Nitric oxide synthase catalyzes the mixed functional oxidation of t-arginine to t-citrulline and nitric oxide (NO5˙) [Stuehr et al., Proc. Natl. Acad. Sci. USA 88, 7773 (1991)]. NO˙ appears to function as either a signaling or an effector molecule depending on the isoform of the enzyme. The constitutive
20   isoform of nitric oxide synthase produces small amounts of NO˙ which activate guanylate cyclase resulting in the formation of cGMP which mediates endothelium-dependent relaxation [Moncada et al., Pharmacol. Reviews 43, 109 (1991)] and neural transmission [Garthwaite, Trends Neurol. Sci. 14, 60 (1991)]. NO˙ is produced in much larger amounts by the cytokine and endotoxin inducible
25   isof rm f nitric oxide synthase, and in macrophages functions as an effector m lecul which appears t mediate the cytotoxic acti ns of macrophages on target cells [Hibbs et al., Nitric Oxide from L-Arginine: A Bioregulatory System, S.

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Moncada and E. Higgs, Eds. Elsevier, NY, pp. 189-223 (1990)]. Since NO˙ is a

potent vasodilator and increases blood flow, and since vasoactive agents (such as

histamine and bradykinin), which stimulate NO˙ production increase both blood

flow and vascular permeability, NO˙ may be a candidate for mediating increases in

5   blood flow and vascular permeability induced by diabetes and elevated glucose

[Pugliese et al., Diabetes/Metabolism Reviews 7, 35 (1991)].

Recently, Interleukin-1 (IL-1) has been shown to induce the expression of the

cytokine inducible isoform of nitric oxide synthase in pancreatic islets. The

production of NO˙ has been proposed to be the effector molecule which mediates

10   IL-1's inhibitory effects on islet function [Southern et al., FEBS. Lett. 276, 42

(1990) and Corbett et al., Biochemical J. 287, 229 (1992)]. Generation of an

IL-1-induced EPR detectable iron-nitrosyl complex, which is prevented by N^G-

monomethyl-t-arginine (NNMA), has been used to confirm the formation of nitric

oxide by islets [Corbett et al., J. Biol. Chem. 266, pp. 21351-21354 (1991)]. Also,

15   the protein synthesis inhibitor, cycloheximide has been shown to block

IL-1-induced nitrite formation, cGMP accumulation, and EPR detectable

iron-nitrosyl complex formation by islets, thus establishing that IL-1 induces

the cytokine inducible isoform of nitric oxide synthase in pancreatic islets [Corbett

et al., Biochemical J. 287, 229 (1992)].

20   The pathogenesis of diabetic complications has been linked to imbalances in

sorbitol, myo-inositol, and 1,2-diacyl-sn-glycerol metabolism, and to

non-enzymatic glycation of cellular and extracellular constituents [Pugliese et al.,

Diabetes/Metabolism 35 Reviews 7, 37 (1991)]. The glycation link is supported by

evidence that aminoguanidine, a nucleophilic hydrazine compound, interferes with

25   the formation of these glycation products and also attenuates the development of

several diabetes-induced vascular [Pugliese et al., Diabetes/Metabolism Reviews 7,

35 (1991); Williams n et al., Diabetes & Metab. 16, 3369 (1990); Soulis-Liparota

et al., Diabetes 40, 1328 (1991)], neural [Kihara et al., Proc. Natl. Acad. Sci. USA

88, 6107 (1991)] and collagen changes [Brownlee et al., New Encl. J. Med. 318,

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1315 (1988) and Brownlee et al., Science 232, 1629 (1986)]. Bucala et al., J. Clin.

Invest. 87, 432 (1991) recently, reported that quenching of NO˙ in vitro by

glycated albumin is attenuated by aminoguanidine (present during exposure of

albumin to glycating agents) and suggested that glycation products may impair

5   endothelium-dependent relaxation by attenuating NO˙ activity.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention a novel method of inhibiting nitric oxide

formation in warm blooded mammals afflicted with acute or chronic

inflammatory diseases is provided. The method comprises administering to warm

10   blooded mammals afflicted with acute or chronic inflammatory diseases a small but

nitric oxide inhibitory effective amount of methyl-, dimethyl-, or

amino-substituted guanidines. These inhibitory compounds are also chemically

named as aminoguanidine, N, N' -diaminoguanidine, methylguanidine and 1,

1-dimethylguanidine.

15   It will be understood that pharmaceutically acceptable salts of these compounds,

e.g., the HCl, HCO_3 and sulfate salts, can also be administered to the host in

accordance with the method of the invention. Inflammation can be conveniently

divided into acute and chronic conditions. Acute inflammation is generally of

relatively short duration and lasts for about a few minutes to about one to two

20   days. Its main characteristics are increased blood flow, exudation of fluid and

plasma proteins (edema) and emigration of leukocytes, predominantly neutrophils.

Chronic inflammation is of longer duration and is associated histologically with the

presence of lymphocytes and macrophages and with proliferation of blood vessels

and connective tissue. Inflammation is manifested by heat, redness, swelling, pain

25   and loss of function. See, e.g., Cotran, Kumar and Robbins, Robbins Pathologic

Basis f Disease, 4th ed., W. B. Saunders Company, pp. 40-41 (1989);

Chandrasoma and Taylor, Concise Pathology, First Edition, pp. 35-44, Appleton &

Lange (1991).

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The causes of inflammation are numerous and include such factors as microbial infecti ns (e.g., bacterial and fungal infections), physical agents such as burns, radiation and trauma, chemical agents such as toxins and caustic substances, necrotic tissue and various types of immunologic reactions.

5   The present invention is directed to the prevention/treatment of a broad spectrum of diseases which may be linked to the production of nitric oxide by leukocytes (neutrophils and macrophages) and other cells of nonhemopoietic origin as distinguished from diseases mediated by immunologic reactions as claimed in copending application Serial No. 07/906,632. The treatment of acute inflammatory

10   disease is illustrated herein in greater detail against endotoxin–induced acute uveitis and generalized vascular leakage and against inflammatory bowel disease.

In these illustrative treatments, standard state–of–the–art animal models are used. The endotoxin–induced uveitis model in the rat is as described by Cousins et al., Exo. Eve Res. 39, 665–676 (1984); Herbort et al., Graefe's Arch. Clin. Exo.

15   Ophthalmol. 226, 553–558 (1988). The rat model for inflammatory bowel disease is a modification of that described by Hirono et al., J. Natl. Cancer Inst. 66, 579– 583 (1981). However, it will be understood that the method of the invention is not limited to the treatment of uveitis or the treatment of inflammatory bowel disease, but includes treatment of other acute and chronic inflammatory diseases as

20   mentioned above. These diseases include but are not limited to diseases such as, for example, acute and chronic infections (bacterial and fungal, including diphtheria and pertussis); acute and chronic bronchitis, sinusitis, and upper respiratory infections, including the common cold; acute and chronic gastroenteritis and colitis; acute and chronic cystitis) and urethritis; acute and chronic dermatitis; acute and

25   chronic conjunctivitis; acute and chronic serositis (pericarditis, peritonitis, synovitis, pleuritis and tendinitis); uremic pericarditis; acute and chronic cholecystitis; acute and chronic vaginitis; drug reactions; insect bites; burns (thermal, chemical, and electrical); and sunburn.

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The present invention is likewise applicable to the treatment of a variety of
inflammat ry disease states including infectious diseases where active infection
exists at any body site, such as in the instance of meningitis. Also included are
conditions such as secondary infections that may occur at a site of antigen

5   deposition that is secondary to a primary infection at a distant body site, and
exemplary specific conditions would include meningitis, encephalitis, arthritis, and
skin conditions such as psoriasis, whether acute or chronic; eczema; contact
dermatitis; poison ivy; poison oak; poison sumac; and like inflammation-mediated
conditions. Also included is the inflammation that results from alterations in

10 leukocyte movement during infection such as adult respiratory distress syndrome
associated with sepsis.

Other inflammatory disease states include those associated with immune disorders
including involvement with T-cell and/or macrophage attachment/recognition, such
as acute and delayed hypersensitivity, graft vs. host disease; auto-immune

15 conditions such as pernicious anemia, Type I diabetes mellitus, rheumatoid arthritis
and multiple sclerosis; antigen-antibody complex mediated diseases including
certain of the secondary infection states listed above; and transplant rejection.
Inflammation due to toxic shock or trauma such as adult respiratory distress
syndrome and reperfusion injury; is likewise included within the scope hereof.

20   DETAILED DESCRIPTION OF THE INVENTION

While the specification concludes with claims particularly pointing out and
distinctly claiming the subject matter regarded as forming the present invention, it
is believed that the invention will be better understood from the following detailed
description of preferred embodiments of the invention taken in conjunction with the

25 accompanying drawings in which briefly:

FIG. 1 is a bar graph which shows the effect of increasing dose of
lipopolysaccharide (LPS; endotoxin purified from Salmonella minnesota) on

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regional ^{125}I–albumin permeation (normalized to contr ls). The fold increase (from
0 t in excess of 4–f ld increase) over controls is shown on the y axis for various
tissues indicated on the x axis at three levels of LPS, namely 50 µg, 100 µg and
200 µg LPS as a divided dose injected into both hind footpads of Lewis rats.

5 FIG. 2 is a bar graph which shows the effect of 35 endotoxin dose on regional
blood flow changes. Blood flow changes in ml/min/g wet weight are shown as a
fold increase (from 0 to in excess of 1.5-fold increase) on the y axis for various
tissues indicated on the x axis at two levels of LPS, namely 100 µg and 200 µg
LPS as a divided dose injected in Lewis rats as in FIG. 1.

10 FIG. 3 in four parts, FIGS. 3A, 3B, 3C and 3D, is a graphical representation which
shows aqueous fluid leukocyte cell counts in Lewis rats treated with 100 µg
LPS. Cell counts of the aqueous fluid (FIG. 3A) and of three types of infiltrating
cells into the aqueous fluid (FIG. 3B, lymphocytes; FIG. 3C, monocytes; and FIG.
3D, PMN) are shown on a logarithmic scale on the y axis for controls (–) and for
15 LPS before (–) and after (+) treatment with 100 mg/Kg aminoguanidine (Ag) on
the x axis. Each filled in circle (●) represents the cell count from an individual
rat.

FIG. 4 is a graphical representation which shows the effect of aminoguanidine on
plasma nitrate/nitrite levels in Lewis rats treated with 100 4g LPS. Plasma
20 nitrate/nitrite levels in µg are shown on the y axis for 20 control (–) and for LPS
before (–) and after (+) treatment with 100 mg/Kg aminoguanidine.

Fig. 5 is a graph demonstrating that aminoguanidine attenuates IL–1–induced PGE$_2$
production by rat islets.

Fig. 6 is a graph demonstrating that aminoguanidine attenuates IL–1–induced PGE$_2$
25 production by purified ß–cells.

Fig. 7 is a graph dem nstrating that exogenous nitric xide stimulates PGE$_2$ production by rat islets.

In order to further illustrate the invention, the following detailed Examples were carried out although it should be understood that the invention is not limited to
5   these specific Examples or the details described therein which are for illustrative and not limitative purposes. The results obtained in these Examples are further shown in Tables 1 to 4 hereinbelow and the accompanying Figures 1 to 4.

EXAMPLE 1

Endotoxin-induced acute uveitis and generalized vascular leakage: inhibition by
10   aminoguanidine

Methods
Animals and materials
Male Lewis rats (-200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed and cared for in accordance with the guidelines of
15   the Washington University Committee for the Humane Care of Laboratory Animals and in accordance with NIH guidelines on laboratory animal welfare. Rats were housed individually, fed standard rat chow (Ralston Purina, Richmond, IN) and water ad libitum, and were on a 12 hour light/dark cycle. Aminoguanidine (hemisulfate), and lipopolysaccharide (LPS; Salmonella minnesota) were purchased
20   from Sigma (St. Louis, MO). ^{125}I and ^{46}Sc microspheres were obtained from NEN Research Products (Boston, MA). 1311 was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

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Induction of LPS-induced vascular injury

LPS was dissolved in steril pyrogen-free 0.9% saline at concentrations of 1 pg/pl, and unless stated otherwise, 50 pl was injected into each hind footpad (100 pg total dose per rat) using metofane anesthesia as described previously by other

5   investigators [Herbort et al., Graefe's Arch. Clin. Exn. Opththalmol. 226, pp. 553-558 (1988); and Hoekzema et al., Invest. Ophthalmol. Vis. Sci. 33, pp. 532-539 (1992)]. Control rats received an equal volume of 0.9% saline. 100 mg/kg body weight aminoguanidine was injected subcutaneously in 30 LPS-treated and in control rats at 0, 3, 6, 12, 18 and within 60 minutes of the initiation of the

10   test, which corresponds to 20-24 hours after LPS injection.

Assessment of vascular function

Regional vascular albumin permeation was quantified by use of an isotope dilution technique based on the injection of bovine serum albumin (BSA) labeled with 2 different iodine isotopes, ^{125}I and ^{131}I [[Pugliese et al., Metabolism 39 pp. 690-697

15   (1990); Pugliese et al., Diabetes 39, pp. 323-332 (1990); and Pugliese et al., Diabetes 39, pp. 312-322 (1990)]. ^{125}I-BSA was used to quantify vascular albumin filtration after 10 min. of tracer circulation while ^{131}1-BSA served as a plasma volume marker for correction of ^{125}I-BSA tissue activity for tracer contained within vessels. Purified monomer IBSA (1 mg) was iodinated with 1

20   mCi of ^{131}1 or ^{125}I by the iodogen, method as previously described [Pugliese et al., Diabetes 39, pp. 323-332 (1990)].

Rats were anesthetized with Inactin (Byk Gulden, Konstanz, FRG) (-100 mg/kg body weight injected i.p.), and core body temperature maintained at 37 ± 0.5°C using heat lamps, a 37°C surgical tray, and, a rectal temperature probe. The left

25   femoral vein, left iliac artery, and right subclavian artery were cannulated with polyethylene tubing (0.58 mm i.d.) filled with heparinized saline (400 V heparin/ml). The femoral vein cannula was used for tracer injection and the subclavian artery cannula was connected to a pressure transducer for blood pressure

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m nitoring. The left iliac artery was connected to a 1 ml syringe attached to a

Harvard Model 940 constant withdrawal pump preset to withdraw at

a constant rate of, 0.055 ml/min. The trachea was intubated and connected to a

small rodent respirator for continuous ventilatory support. Microspheres were

5   injected into the left ventricle through a carotid-artery cannula.

At time 0, ^{125}I-albumin was injected and the withdrawal pump was started

simultaneously. Eight min. after time 0, 131I-BSA was injected, followed by the

microspheres. At the 10 min mark, the heart was excised to stop all blood flow,

the withdrawal pump was stopped simultaneously, and various tissues were

10 sampled for gamma spectrometry.

The left eye was dissected as previously-described [Pugliese et al., Diabetes 39,

pp. 323-332 (1990); and Pugliese et al., Diabetes 39, pp. 312-322 (1990)] and all

tissue samples and arterial plasma samples were weighed, then counted in a gamma

spectrometer. A quantitative index of ^{125}I-BSA tissue clearance was calculated as

15 previously described in [Pugliese et al., Metabolism 39, pp. 690-697 (1990);

Pugliese et al., Diabetes 39, pp. 323-332 (1990); Pugliese et al., and

Diabetes 39, pp. 312-322 (1990)] and expressed as μg plasma/g tissue wet

weight/min. Briefly, ^{125}I-BSA tissue activity was corrected for tracer contained

within the tissue vasculature by multiplying ^{125}I-BSA activity in the tissue by the

20 ratio of ^{125}I-BSA/131I-BSA activities in the arterial plasma sample obtained at the

end of the test. The vascular-corrected ^{125}I-BSA tissue activity was divided by the

time-averaged ^{125}I-BSA plasma activity (obtained from a well mixed sample of

plasma taken from the withdrawal syringe) and by the tracer circulation time (10

min) and then normalized per g tissue wet weight. To calculate blood flow, total

25 activity of ^{46}Sc in each ocular tissue was divided by the activity of ^{46}Sc in the

reference blood sample obtained from the withdrawal pump syringe, multiplied by

the pump withdrawal rate, and expressed as ml/g tissue/min [Pugliese et al.

Diabetes 39, pp. 323-332 (1990) and Pugliese et al., Diabetes 39, pp. 312-322

(1990)]. Other n n-ocular tissues (aorta, kidney, skin, skeletal muscle, sciatic

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nerv) also were sampled to assess if LPS induced a generalized vascular
dysfunction.

Assessment of cells in aqueous humor

Aqueous fluid was collected from the left eye only (the right carotid artery was

5 ligated for the blood flow studies and this eye was not used for function studies)
using a 100 µg heparinized capillary tube and placed in a microfuge tube. Well
mixed aliquots (2 µl) were spread on a siliconized glass slide, air dried, stained
with Wright's stain, and the total number of cells and a differential cell count were
performed using a Leitz orthoplan light microscope.

10 ### Plasma nitrate/nitrite measurements

Anticoagulated (heparin) plasma samples were centrifuged at 7500 rpm for 1 hour
at 4°C using a 10,000 5 molecular weight cut off Centricon filter (Amicon,
Beverly, MA). Plasma nitrate was enzymatically reduced to nitrite using
Aspergillus niger nitrate reductase (Sigma, St. Louis, MO). Briefly, the sample

15 was incubated with 40 µM NADPH and 14 mU of enzyme in a final volume of 50
41 of 20 mM Tris, pH 7.6; the reaction was terminated after 5 minutes at 20°C by
dilution with 50 µl of water followed by addition of 10 µl of freshly prepared
DAN reagent (0.05 mg/ml in 0.6 M HCl) for determination of nitrite. The DAN
assay is a modification of the conventional method of Damiani and Burini for the

20 fluorometric determination of nitrite [Talanta 33, 649–652 (1986)].
2,3-Diaminonaphthalene (DAN) is reacted with nitrite under acidic conditions to
form 1-(H)-naphthotriazole, a fluorescent product. After a 10 minute incubation
at 20°C, the reaction was terminated with 5 µl of 2.8 N NaOH, which maximizes
the intensity of the fluorescent signal. Formation of the

25 2,3-diaminonaphthotriazole was measured using a Pandex (IDEXX Laboratories,
Inc., Westbrook, ME) fluorescent plate reader with excitation at 365 nm and
emission read at 450 nm with a gain setting at 100%. Plasma nitrite levels were
calculated by first subtracting the value of the enzyme blank (nitrate reductase plus
NADPH) from the experimental reading, then calculating the value using a

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standard curve for nitrite to which NADPH was added.

Statistical analysis

All results are expressed as means ± standard deviations. Overall differences

among test groups for each parameter were first assessed by the Van der

5   Waerden test, and individual pair-wise group comparisons were evaluated by at

least square means analysis only if the Van der Waerden test was significant at

$p < 0.05$ for a given parameter. A nonparametric Blom transformation of

all data was performed prior to assessment of individual pair-wise group

differences.

10   ## Results

Generalized vascular leakage of ^{125}I-albumin

In preliminary tests, 50, 100, and 200 pg LPS were injected either as a single

injection into one hind footpad or as a divided dose injected into both hind

footpads of Lewis rats. In general, increases in ^{125}I albumin leakage were greater

15   for the divided dose of LPS versus a single injection. Figure 1 shows changes in

[125]I-albumin leakage expressed as a fold increase over control values for LPS given

as a divided dose. Except for sciatic nerve, maximal increases in ^{125}I-albumin

leakage normalized to control values were observed with 100 µg LPS and this dose

was selected for subsequent tests.

20   Table 1 shows changes in ^{125}I-albumin permeation resulting from the injection of

100 pg LPS (50 µg/footpad) into footpads of Lewis rats. The Van der Waerden

test indicated highly significant group differences for ^{125}I-albumin leakage in the

retina, anterior uvea, choroid/sclera, and aqueous fluid ($p < 0.0001$), while changes

were absent in brain. At the dose and frequency used, aminoguanidine attenuated

25   the [125] LPS-induced ^{125}I-albumin leakage by −75% in the retina, sciatic nerve, and

aorta, by −50% in the aqueous fluid and anterior uvea, and by −30% in the

posterior uvea, without affecting ^{125}I-albumin leakage in controls.

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Figure 2 shows changes in regional blood flows expressed as a fold increase over
control values for LPS administered as a divided dose. Regional blood flows

increased with increasing dose of LPS in the anterior and posterior uveal
vasculatures and in the heart, plateaued in the-retinal and sciatic nerve at 100 pg
5 LPS, and was unaffected by LPS in the kidney when measured 20 hours after LPS
injection. The Van der Waerden test indicated significant group differences for
blood flow in the anterior uvea and choroid/sclera but not in the retina, sciatic
nerve, brain, heart, and kidney (Table 2). LPS increased blood flow ~40% in the
anterior uvea and ~25% in the choroid-sclera and these increases were prevented
10 by aminoguanidine.

Gravimetric and hemodynamic parameters

All gravimetric and hemodynamic parameters, including body weight, mean arterial
blood pressure, cardiac output, cardiac index, total peripheral
resistance, GFR (normalized either to whole kidney or g kidney wet weight),
15 glomerular filtration fraction, and renal vascular resistance, were unaffected by the
injection of 100 μg LPS (Table 3).

Aqueous fluid leukocyte cell counts

Number of cells in a well mixed 2 μl sample of aqueous fluid from controls was
low, ranging from no cells in 6 animals to ~25 cells in 2 rats (Figure 3); the
20 median value was ~5 cells/2 μl fluid and was unaffected by aminoguanidine
treatment in controls. As shown in Figure 3A, 100 μl LPS significantly increased
the number of cells in 2 μl of aqueous fluid. Figure 3 also shows the types of
infiltrating cells into the aqueous fluid following LPS injection. Virtually all
cells in the aqueous fluid of controls (± aminoguanidine) were lymphocytes. In
25 aqueous fluid of rats treated with 100 μg LPS, lymphocytes (Figure 3B),
monocytes (Figure 3C), and PMNs (Figure 3D) increased significantly with the
largest increase observed for PMNs. Aminoguanidine reduced the total number of

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cells in the aqueous fluid of LPS-treated rats, including ~90% reduction in the
number of PMNs.

Plasma nitrate/nitrite measurement

Plasma nitrate/nitrite levels were 17.9 ± 8.6 μM for controls and were increased

5   ~65% in LPS-treated rats (Figure 4). Aminoguanidine treatment prevented the
LPS-induced increases in plasma nitrate/nitrite levels.

It is seen from the above results that aminoguanidine markedly attenuated
manifestations of endotoxin-induced acute uveitis (vascular-leakage,
increased blood flow, and exudation of leukocytes into the aqueous fluid) as well

10 as elevated plasma nitrate/nitrite levels. Aminoguanidine also significantly
attenuated or completely prevented systemic effects of endotoxin on vascular
leakage, i.e., edema (an important manifestation/consequence of
inflammation) in nerve, aorta, and small intestine.

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Table 1: Effects of LPS (100 μg) and aminoguanidine (100 mg/kg) on regional
^{125}I–albumin permeation (μg plasma/min/g wet weight)

	control	LPS	LPS + aminoguanidine
number of rats	14	19	11

eye
  aqueous fluid
  anterior uvea
  posterior uvea
  retina
	65 ± 23[a]
	270 ± 53
	258 ± 75
	66 ± 14
		294 ± 81[b]
		611 ± 110[b]
		612 ± 110[b]
		154 ± 32[b]
			204 ± 53[b,f]
			423 ± 67[b,e]
			497 ± 96[b,f]
			86 ± 23[e]

sciatic nerve	62 ± 14	167 ± 35[b]	91 ± 20[c,e]
aorta	75 ± 26	245 ± 64[b]	114 ± 43 [d,e]
skeletal muscle	44 ± 17	62 ± 11	51 ± 10
skin	110 ± 27	209 ± 73[b]	186 ± 49[c]
brain	23 ± 11	24 ± 11	25 ± 15
heart	621 ± 57	585 ± 116	624 ± 86
small intestine	377 ± 112	567 ± 247[d]	375 ± 100[g]
kidney	612 ± 212	1,173 ± 407[b]	1,578 ± 251[b,f]

Male Lewis rats were treated with 100 μg LPS (50 pg in each hind footpad) ± 100
mg/kg body weight aminoguanidine hemisulfate (at the time of injection of LPS
and 3, 6, 12, 18. and 20–24 hours thereafter). LPS was injected at noon and the
animals were sacrificed the following morning.

[a] values are mean ± SD
Significantly different from controls by least square means analysis:
[b] p<0.0001; [c] p<0.001; [d] p<0.05

Significantly different from LPS–treated rats least square means analysis:
[e] p<0.0001; [f] p<0.005; [g] p<0.05

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Table 2: Effects of LPS (100 μg) and aminoguanidine (100 mg/kg) on regional
blood flows (ml/min/g wet weight)

	control	LPS	LPS + aminoguanidine
number of rats	11	13	9
eye anterior uvea posterior uvea retina	1.8 ± 0.4 3.4 ± 0.6 0.42 ± 0.03	2.5 ± 0.6[b] 4.3 ± 0.9[c] 0.45 ± 0.07	1.9 ± 0.3[d] 3.7 ± 0.4 0.42 ± 0.04
sciatic nerve	0.07 ± 0.01	0.08 ± 0.03	0.08 ± 0.03
brain	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
heart	4.9 ± 1.4	6.1 ± 1.2	5.4 ± 1.7
kidney	4.8 ± 0.9	4.7 ± 0.6	4.9 ± 0.6

Male Lewis rats were treated with 100 μg LPS (50 μg in each hind footpad) ± 100
mg/kg body weight aminoguanidine hemisulfate (at the time of injection of LPS
and 3, 6, 12, 18, and 20–24 hours thereafter). LPS was injected at noon and the
animals were sacrificed the following morning.

[a] values are mean ± SD

Significantly different from controls by least square means analysis:
[b] $p<0.0001$; [c] $p<0.005$

Significantly different from LPS-treated rats least square means analysis:
[d] $p<0.005$

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Table 3:   Effects f LPS (100 µg) and aminoguanidine (100 mg/kg) on gravimetric and hemodynamic parameters

	control	LPS	LPS + aminoguanidine
number of rats	5	6	7
body weight (g)	237 ± 15[a]	225 ± 9	229 ± 12
mean arterial blood pressure (mm Hg)	120 ± 9	121 ± 9	124 ± 10
cardiac output (ml/min)	81 ± 9	75 ± 4	77 ± 3
cardiac index (ml/min/100 g b.w.)	343 ± 19	334 ± 13	336 ± 23
total peripheral resistance (mm Hg/ml/min)	1.42 ± 0.10	1.55 ± 0.14	1.54 ± 0.11
GFR (ml/min/whole kidney)	0.96 ± 0.07	0.97 ± 0.06	0.95 ± 0.14
GFR (ml/min/g kidney)	0.87 ± 0.07	0.90 ± 0.11	0.87 ± 0.15
filtration fraction (GFR/renal blood flow)	0.34 ± 0.08	0.34 ± 0.05	0.31 ± 0.06
renal vascular resistance (mm Hg/ml/min)	0.97 ± 0.07	0.97 ± 0.06	0.95 ± 0.14

Male Lewis rats were treated with 100 µg LPS (50 µg in each hind footpad) ± 100 mg/kg body weight aminoguanidine hemisulfate (at the time of injection of LPS and 3, 6, 12, 18, and 20–24 hours thereafter). LPS was injected at noon and the animals were sacrificed the following morning.

[a] values are mean ± SD

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EXAMPLE 2

End toxin-induced acute uveitis and generalized vascular leakage: inhibition by methylguanidine

Methods

5 ### Animals and Materials

Male Lewis rats (~200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals in accordance with NIH guidelines on laboratory animal welfare. Rats were housed

10 individually, fed standard rat chow (Ralston Purina, Richmond, IN) and water ad libitum, and were on a 12 hour light/dark cycle. Methylguanidine (hydrochloride) and lipopolysaccharide (LPS; Salmonella minnesota) were purchased from Sigma (St. Louis, MO). ^{125}I was obtained from NEN Research Products (Boston, MA). ^{131}I was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

15 ### Induction of LPS-Inducted Vascular Injury

LPS was dissolved in sterile pyrogen-free 0.9% saline at concentrations of 1 $\mu g/\mu l$, and 50 μl was injected into each hind footpad (100 μg total dose per rat) using metofane anesthesia as described previously by other investigators [Herbort et al., Graefe's Arch. Clin. Exp. Ophthalmol. 226, pp. 553-558 (1988); and

20 Hoekzema et al., Invest. Ophthalmol. Vis. Sci. 33, pp. 532-539 (1992)]. Control rats received an equal volume of 0.9% saline. 50 mg/kg body weight methylguanidine was injected subcutaneously in LPS-treated and in control rats at 0, 3,6,12,18 and 20-24 hours after LPS injection. Animals were killed for the vascular function studies 20 to 24 hours after LPS injection.

25 ### Assessment of Vascular Function

Regional vascular albumin permeation was quantified by use of an isotope dilution technique based on the injecti n f bovine serum albumin (BSA) labeled with 2 different iodine isot pes, ^{125}I and ^{131}I [Pugliese et al., Metabolism 39, pp. 690-697

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(1990); Pugliese et al., Diabetes 39, pp. 323-332 (1990); and Pugliese et al.,

Diabetes 39, pp. 312-322 (1990)]. ^{125}I-BSA was used to quantify vascular

albumin filtration after 10 minutes of tracer circulation, while ^{131}I-BSA served as a

plasma volume marker for correction of ^{125}I-BSA tissue activity for tracer

5   contained within vessels. Purified monomer BSA (1 mg) was iodinated with 1

mCi of ^{131}I or ^{125}I by the iodogen method as previously described in Pugliese et al.,

Diabetes 39, pp. 323-332 (1990).

Rats were anesthetized with Inactin (Byk Gulden Konstanze, FRG) (~100 mg/kg

body weight injected i.p.), and core body temperature maintained at 37 ± 0.5°C

10   using heat lamps, a 37°C surgical tray, and a rectal temperature probe. the left

femoral vein, left iliac artery, and right subclavian artery were cannulated with

polyethylene tubing (0.58 mm i.d.) filled with heparinized saline (400 U

heparin/ml). The femoral vein cannula were used for tracer injection and the

subclavian artery cannula was connected to a pressure transducer for blood pressure

15   monitoring. The left iliac artery was connected to a 1 ml syringe attached to a

Harvard Model 940 constant withdrawal pump preset to withdraw at a constant rate

of 0.055 ml/min. The trachea was intubated and connected to a small rodent

respirator for continuous ventilatory support.

At time 0, ^{125}I-albumin was injected i.v. and the withdrawal pump was started

20   simultaneously. Eight minutes after time 0, ^{131}I-BSA was injected. At the 10

minute mark, the heart was excised to stop all blood flow, the withdrawal pump

was stopped simultaneously, and various tissues were sampled for gamma

spectrometry. The left eye was dissected as previously described [Pugliese et al.,

Diabetes 39, pp. 323-332 (1990) and Pugliese et al., Diabetes 39, pp.l 312-322

25   (1990)] and all tissue samples and arterial plasma samples were weighed, then

counted in a gamma spectrometer. A quantitative index of ^{125}I-BSA tissue

clearance was calculated as previously described [Pugliese et al., Metabolism 39,

pp.l 690-697 (1990); Pugliese et al., Diabetes 39, pp. 323-332 (1990); and

Pugliese et al., Diabetes 39, pp. 312-322 (1990)] and expressed as μg plasma/g

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tissue wet weight/min. Briefly, ^{125}I-BSA tissue activity was corrected for tracer contained within the tissue vasculature by multiplying ^{125}I-BSA activity in the tissue by the ratio of ^{125}I-BSA/131I-BSA activities in the arterial plasma sample obtained at the end of the test. The vascular corrected ^{125}I-BSA tissue activity was

5   divided by the time-averaged ^{125}I-BSA plasma activity (obtained from a well-mixed sample of plasma taken from the withdrawal syringe) and by the tracer circulation time (10 minutes) and then normalized per g tissue wet weight. Other non-ocular tissues (aorta, kidney, skin, skeletal muscle, sciatic nerve) also were sampled to assess if LPS induced a generalized vascular dysfunction.

10   **Statistical Analysis**

All results are expressed as means ± standard deviations. Overall differences among test groups for each parameter were assessed by Students' test.

Generalized Vascular Leakage of ^{125}I-Albumin

Table 4 shows changes in ^{125}I-albumin permeation resulting from the injection of

15   100 μg LPS (50 μg/footpad) into footpads of Lewis rats. Highly significant group differences for ^{125}I-albumin leakage were evident in the aqueous fluid, anterior uvea, choroid/sclera, retina, sciatic nerve, aorta, and kidney ($p < 0.0001$), while smaller increases were observed in the skin and skeletal muscle. No LPS-induced changes were present in brain, heart, or small intestine. At the dose and frequency

20   used, methylguanidine attenuated the LPS-induced ^{125}I-albumin leakage in aqueous fluid, anterior uvea, and kidney and prevented the LPS-induced increases in albumin leakage in the choroid/sclera, retina, sciatic nerve, aorta, and skin.

It is seen from the above results that methylguanidine (at approximately one-half of the dose of aminoguanidine used in Example 1) was as effective as (or more

25   effective than) aminoguanidine in preventing endotoxin-induced vascular leakage (an important manifestation/consequence of inflammation) in uveal tissue as well as in the sciatic nerve, aorta, skeletal muscle, skin, and kidney.

20

Similar results as obtained in Examples 1 and 2 can be btained by substituting N,N'-diaminoguanidine or 1,1-dimethylguanidine for equivalent amounts of aminoguanidine or methylguanidine, respectively, in said Examples.

Table 4: Effects of LPS μg) and methylguanidine (mg) on regional
^{125}I-albumin permeation[a]

	control	LPS	LPS + aminoguanidine
number of rats	5	8	4
aqueous	154 ± 100	495 ± 283[a]	335 ± 30[c]
anterior uvea	266 ± 52	618 ± 106[a]	383 ± 32[c,e]
choroid/sclera	298 ± 67	582 ± 77[a]	357 ± 50[d]
retina	68 ± 15	168 ± 28[a]	67 ± 7[d]
sciatic nerve	71 ± 14	187 ± 32[a]	69 ± 3[d]
aorta	75 ± 24	211 ± 43[a]	102 ± 8 [d]
skeletal muscle	54 ± 19	103 ± 46[c]	63 ± 11
skin	99 ± 29	224 ± 65[b]	106 ± 16[f]
brain	20 ± 4	25 ± 6	19 ± 5
heart	623 ± 43	699 ± 58	629 ± 82
small intestine	414 ± 40	559 ± 147	423 ± 188
kidney	635 ± 239	1,561 ± 221[a]	1,042 ± 253[c,e]

[a] μg plasma/g wet weight/min; values are mean ± SD.

Male, Lewis rats were treated with 100 μg LPS (50 pg in each hind footpad) ± 50 mg/kg body weight methylguanidine hydrochloride (at the time of injection of LPS and 3, 6, 12, 18, and 20-24 hours thereafter). LPS was injected at noon and the animals were sacrificed the following morning.

Significantly different from untreated controls:
[a] $p<0.0001$; [b] $p<0.005$; [c] $p<0.05$

Significantly different from LPS:
[d] $p<0.001$; [e] $p<0.005$; [f] $p<0.01$

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Inflammation conditi ns are characterized by the accumulation of polymorphonuclear leukocytes, macrophages and monocytes at the site of injury. Macrophage and monocytes have been shown to produce high levels of inflammatory mediators at the sites of inflammation. In the carrageenin and

5   carrageenin induced paw oedema models of inflammation the production of nitric oxide has also been demonstrated [Ialenti et al. European J. of Pharacol. 211:177-182 (1991)]. Macrophage are believed to be the cellular source of inducible nitric oxide synthase (iNOS) and the cell type that produces nitric oxide at the site of inflammation. Macrophage release of cytokines during the inflammatory reaction

10   may also induce the expression of iNOS by other cellular sources. Proinflammatory prostaglandin (PGs) and thromboxane production has also been demonstrated in these animal models of inflammation [Masferrer et al., Proc. Natl. Acad. Sci. USA 91:3228-3232 (1994)]. The cellular source being macrophage and monocytes and fibroblasts. Cyclooxygenase (COX) catalyzes the first enzymatic

15   reaction in the production of prostaglandins, prostacyclin, and thromboxane. Two isoforms of COX have been demonstrated. Constitutive COX (COX-1) is found in most tissues and is responsible for the physiological production of prostaglandins (Dewitt, Biochim. Biophys. Acta 3:121-124 (1991)]. Prostaglandins, producted by COX-1, play a prominent role in the normal physiological function of the stomach

20   and kidney [Dewitt, supra]. Expression of other isoforms of COX is inducible by cytokines. This isoform, inducible COX (or COX-2) is believed to be responsible for the production of PGs under conditions of inflammation and injury [Fletcher et al., J. Biol. Chem. 267:4338-4344 (1992)]. Recent studies have shown the expression of COX-2 at the level of mRNA, protein, and enzymatic activity in the

25   rat air pouch model of inflammation [Masferrer et al., supra]. During this inflammation the production of PGs appears to be entirely the result of COX-2 activity [Masferrer et al., supra].

Autoimmune diabetes is an inflammatory disease that is characterized by macrophage and lymphocyte invasion f islets, followed by ß-cell death [Bach,

30   Endocrine Rev. 15:516-542 (1994)]. Cytokines released during this lymphocytic

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infiltration have been proposed to participate in ß-cell destruction during the

devel pment of autoimmune diabetes [Corbett et al., Diabetes 41:897-903 (1992)].

Treatment of rat islets with the cytokine IL-1, induces a potent inhibition of

insulin secretion that is followed by ß-cell destruction. Nitric oxide appears to

5   mediate the inhibitory and destructive effects of IL-1 on islets. Inhibitors of nitric

oxide synthase (aminoguanidine and monomethyl-L-arginine) completely prevent

IL-1 induced inhibition of insulin secretion and islet destruction [Corbett et al.,

Autoimmunity 15:145-153 (1993); Corbett et al., Diabetes 41:552-556 (1991)].

IL-1 also induces the expression of COX-2 and the production of PGE_2 by islets

10   [Corbett et al., Biochemistry 32, 13767-13770 (1993)]. PGE_2 production by islets

is attenuated by the nitric oxide synthase inhibitors NMMA, and aminoguanidine

(AG) [Corbett et al., *supra*]. These findings suggest that cytokines, nitric oxide,

and nitric oxide stimulated PGs production play key roles in the development of

autoimmune diabetes, in a manner similar to inflammatory conditions where the

15   production of nitric oxide, the release of cytokines, and PGs release have been

demonstrated.

EXAMPLE 3

Additional experimental data were developed that relate to the effect of

aminoguanidine on prostaglandin production as measured by cyclooxygenase-2

20   (COX-2) activity. COX-2 has been identified as a primary producer of

prostaglandins during inflammation.

The data is set forth in Figs. 5-7 and is described briefly below. This data

demonstrates the interactions of the nitric oxide and prostaglandin pathways. Nitric

oxide appears to directly activate the enzymatic activity of COX-2 resulting in the

25   overproduction of PGE_2. Inhibitors of nitric oxide synthase (AG, and NMMA)

attenuate nitric oxide stimulated production of PGE_2. These inhibitors do not have

inhibitory ffects on the expression of either iNOS r COX-2(9). Exogen us

production of nitric oxide (spontaneously released by nitric oxide donor compound,

SIN-1) stimulates the production of PGE$_2$ by islets, further supporting the direct activation of COX by nitric oxide (Fig. 7). These studies support the use of iNOS inhibitors, NMMA and AG for the inhibition of PGs production under inflammatory conditions such as acute and chronic inflammation, arthritis,

5   inflammatory bowel disease, injury, etc. The inhibitors function by preventing nitric oxide activation of COX-2.

Referring now to Fig. 5, isolated rat islets were incubated for 24 h with 5 units/ml IL-1, or 0.5 mM AG as indicated. The supernatant was removed and PGE$_2$ and nitrite formation were determined. Results demonstrate that AG attenuates IL-1-

10   induced PGE$_2$ and completely prevents IL-1-induced nitrite formation by rat islets. These findings indicate that IL-1-induces COX-2 and iNOS expression and that nitric oxide directly activates COX-2.

In a further experiment the results of which are set forth in Fig. 6, purified ß-cells were incubated for 24 h with 5 unites/ml IL-1, 0.5 mM aminoguanidine, or 1 µM

15   Actinomycin D as indicated. The supernatant was removed and PGE$_2$ and nitrite formation were determined. These results further demonstrate that aminoguanidine attenuates IL-1-induced PGE$_2$ production, and completely prevents IL-1-induced nitrite formation by ß-cells. The transcriptional inhibitor actinomycin D completely prevents PGE$_2$ and nitrite formation by ß-cells, indicated the

20   requirement for mRNA transcription. These findings indicate that IL-1-induces COX-2 and iNOS expression and that nitric oxide directly activates COX-2.

As further corroboration of the activity and interaction of nitric oxide in COX and PGE$_2$ production, rat islets were pretreated with 5 units/ml IL-1 or IL-1 and actinomycin D (1µM) for 18 h. The islets were washed and then cultured for 2 h

25   with 30 µM arachidonic acid (COX substrate) in the presence or absence of SIN-1 (1 mM; nitric oxide donor compound) and hemoglobin (Hb; scavenger of nitric oxide) as indicated. The results are presented in Fig. 7 and demonstrate that nitric oxide, released spontaneous by SIN-1, stimulates the activity of both constitutive

COX (COX-1; control and IL-1+Act D treatment) and inducible COX (COX-2; IL-1 treated group). Hemoglobin attenuates SIN-1 stimulated PGE_2 formation indicating that the effects of SIN-1 on COX activity is mediated by nitric oxide.

EXAMPLE 4

5   Inhibition by aminoguanidine of the vascular injury in inflammatory bowel disease was evaluated in the rat.

Inflammatory bowel disease was induced by feeding male Sprague-Dawley rats pulverized normal rat chow containing 5% dextran sodium sulfate (DSS), mol wt —40 – 50,000. This model is reported to induce loose stools/diarrhea in all animals

10   within 14 days which is followed by extensive ulceration of the colon and eventually by development of carcinomas of the colon (65% of animals by 134 days) [Hirono et al., J. Natl. Cancer Inst. 66, 579-583 (1981)]. Thus the model reproduces many of the characteristic features of chronic ulcerative colitis in humans, including the development of cancer.

15   **Methods**
    Animals and materials
    Male Lewis rats (-200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed and cared for in accordance with the guidelines of the Washington University Committee for the Humane Care of Laboratory Animals

20   and in accordance with NIH guidelines on laboratory animal welfare. Rats were housed individually, fed standard rat chow (Ralston Purina, Richmond, IN) and were on a 12 hour light/dark cycle. Aminoguanidine (hemisulfate) was purchased from Sigma (St. Louis, MO). [125]I and [46]Sc microspheres were obtained from NEN Research Products (Boston, MA).

25   **Induction of Inflammatory-Bowel-Disease vascular injury**
    Based on pilot studies, 4 groups of rats were prepared as follows:
    1. Control rats fed normal rat chow (n=9),
    2. Rats fed chow containing 5% DSS (n=8),

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3. Rats fed chow with 5% DSS + aminoguanidine hemisulfate added to the drinking water in a concentration t provide a daily dose of 25 mg/kg body weight/day (n=5), and

4. Rats fed chow with 5 DSS% and given 25 mg aminoguanidine hemisulfate/kg
5 body weight/day by subcutaneous injection (n=4).

After 7 days on the DSS supplemented diet, the rats were anesthetized for assessment of vascular permeability in the cecum and large bowel. Vascular permeability was quantified by injection of radiolabeled albumin as described below.

10 __Assessment of vascular function__

Regional vascular albumin permeation was quantified by use of an isotope dilution technique based on the injection of bovine serum albumin (BSA) labeled with 2 different iodine isotopes, ^{125}I and ^{131}I [[Pugliese et al., Metabolism 39 pp. 690-697 (1990); Pugliese et al., Diabetes 39, pp. 323-332 (1990); and Pugliese et al.,

15 Diabetes 39, pp. 312-322 (1990)]. ^{125}I-BSA was used to quantify vascular albumin filtration after 10 min. of tracer circulation while ^{131}I-BSA served as a plasma volume marker for correction of ^{125}I-BSA tissue activity for tracer contained within vessels. Purified monomer IBSA (1 mg) was iodinated with 1 mCi of ^{131}I or ^{125}I by the iodogen, method as previously described [Pugliese et al.,

20 Diabetes 39, pp. 323-332 (1990)].

Rats were anesthetized with Inactin (Byk Gulden, Konstanz, FRG) (-100 mg/kg body weight injected i.p.), and core body temperature maintained at $37 \pm 0.5°C$ using heat lamps, a 37°C surgical tray, and, a rectal temperature probe. The left femoral vein, left iliac artery, and right subclavian artery were cannulated with

25 polyethylene tubing (0.58 mm i.d.) filled with heparinized saline (400 V heparin/ml). The femoral vein cannula was used for tracer injection and the subclavian artery cannula was connected to a pressure transducer for blood pressure

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monitoring. The left iliac artery was connected to a 1 ml syringe attached t a Harvard Model 940 constant withdrawal pump preset to withdraw at a constant rate of, 0.055 ml/min. The trachea was intubated and connected to a small rodent respirator for continuous ventilatory support.

5   At time 0, ^{125}I-albumin was injected and the withdrawal pump was started simultaneously. Eight min. after time 0, 131I-BSA was injected, followed by the microspheres. At the 10 min mark, the heart was excised to stop all blood flow, the withdrawal pump was stopped simultaneously, and various tissues were sampled for gamma spectrometry.

10   All tissue samples and arterial plasma samples were weighed and then counted in a gamma spectrometer. A quantitative index of ^{125}I-BSA tissue clearance was calculated as previously described in [Pugliese et al., Metabolism 39, pp. 690-697 (1990); Pugliese et al., Diabetes 39, pp. 323-332 (1990); Pugliese et al., and Diabetes 39, pp. 312-322 (1990)] and expressed as μg plasma/g tissue wet

15   weight/min. Briefly, ^{125}I-BSA tissue activity was corrected for tracer contained within the tissue vasculature by multiplying ^{125}I-BSA activity in the tissue by the ratio of ^{125}I-BSA/131I-BSA activities in the arterial plasma sample obtained at the end of the test. The vascular-corrected ^{125}I-BSA tissue activity was divided by the time-averaged ^{125}I-BSA plasma activity (obtained from a well mixed sample of

20   plasma taken from the withdrawal syringe) and by the tracer circulation time (10 min) and then normalized per g tissue wet weight.

Results

The vascular permeability changes induced by 5% DSS and the effects of aminoguanidine on them are summarized in Table 5. Vascular leakage was

25   increased approximately 2 fold by 5% DSS in the cecum and in all portions of the colon. These increases were markedly attenuated in the cecum, proximal colon, and mid-colon by oral or subcutaneous aminoguanidine. The effects of

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amin guanidine on vascular leakage of albumin in the distal colon were less impressive, although in animals given aminoguanidine by injection, leakage was substantially reduced (p=0.065). The reason oral aminoguanidine was less effective on permeability changes in the distal colon may be due to absorption of the drug in

5　the proximal segments of bowel with insufficient amounts remaining to impact on leakage in the distal colon.

These results show that aminoguanidine given orally or by subcutaneous injection is efficacious in attenuating vascular damage in an animal model of inflammatory bowel disease. Since increased vascular leakage is a characteristic feature of actute

10　and chronic inflammation and contributes to the diarrhea associated with the disease, these findings support the claim that aminoguanidine may be useful in the treatment of inflammatory bowel disease in human subjects.

TABLE 5:　Effects of aminoguanidine[a] on vascular leakage into large bowel in rats given 5% DSS[b] in the diet for 7 days

	Cecum	Proximal colon	Mid-colon	Distal colon
Control (n=9)	218 ± 45[c]	220 ± 47	247 ± 64	264 ± 35
5% DSS (n=8)	458 ± 68[d]	501 ± 107[d]	464 ± 98[d]	562 ± 198[d]
+Ag Oral (n=5)	281 ± 89[e]	249 ± 94[e]	269 ± 78[e]	523 ± 237
+Ag Injection (n=4)	274 ± 63[e]	222 ± 87[e]	209 ± 55[e]	319 ± 88

[a]　25 mg/kg body weight per day in drinking water (oral)
20　　or by subcutaneous injection

[b]　dextran sodium sulfate, (mol. wt. = 40,000-50,000)

[c]　mean ± SD of albumin permeation expressed as
　　μg plasma/g wet wt/min

[d]　significantly different from control: p<0.01

25　[e]　significantly different from 5% DSS: p<0.01

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The inhibit rs of nitric oxide formation described herein can be used for
administration t warm blooded mammals by conventional means, preferably in
formulations with pharmaceutically acceptable diluents and carriers. The amount
of the active inhibitor to be administered must be an effective amount, that is, an

5   amount which is medically beneficial but does not present toxic effects which
overweigh the advantages which accompany its use. It would be expected that the
adult human daily dosage would normally range upward from about one milligram
per kilo of body weight of the drug. Suitable routes of administration include,
where appropriate, topical delivery via salves, ointments and solutions; or locally

10   through suppositories, pessaries, and the like; orally in the form of capsules,
tablets, syrups, elixirs and the like; and parenteral administration, e.g.,
intravenously, intraperitoneally or subcutaneously. Intravenous administration of
the drug in aqueous solution such as physiologic saline is illustrative. Appropriate
formulations of the drug in pharmaceutically acceptable diluents and carriers in

15   therapeutic dosage form can be prepared by reference to general texts in the field
such as, for example, Remington's Pharmaceutical Sciences, Ed. Arthur Osol. 16th
ed., 1980, Mack Publishing Co., Easton, PA.

Various other examples will be apparent to the person skilled in the art after
reading the present disclosure without departing from the spirit and scope of the

20   invention. It is intended that all such examples be included within the scope of the
appended claims.

WHAT IS CLAIMED IS:

1 1.   A method of inhibiting nitric oxide production in a warm blooded mammal
2 afflicted with the physiological conditions manifested by an acute or chronic
3 inflammatory disease or condition which comprises administering to said mammal
4 a nitric oxide inhibitory effective amount of an inhibitory compound selected from
5 the group consisting of aminoguanidine, N,N'-diaminoguanidine, methylguanidine
6 and 1,1-dimethylguanidine.

1 2.   The method of Claim 1 in which the inhibitory compound is
2 aminoguanidine.

3 3.   The method of Claim 1 in which the inflammatory disease or condition is
4 treatable by topical/local administration of said inhibitory compound.

1 4.   The method of Claim 3 in which the inflammatory disease or condition
2 includes but is not limited to a member selected from the group consisting of acute
3 and chronic vaginitis; arthritis; insect bites; burns (thermal, chemical, and
4 electrical); sunburn; acute and delayed hypersensitivity; skin conditions such as
5 psoriasis, whether acute or chronic; eczema; contact dermatitis; poison ivy; poison
6 oak; and poison sumac.

1 5.   The method of Claim 1 in which the inflammatory disease or condition is
2 treatable by oral administration of said inhibitory compound.

1 6.   The method of Claim 5 in which the inflammatory disease or condition
2 includes but is not limited to a member selected from the group consisting of acute
3 and chronic gastroenteritis and colitis; arthritis; acute and chronic cystitis and
4 urethritis; and inflammatory bowel/Crohn's disease.

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1 7.   The method of Claim 1 in which the inflammat ry disease or condition is
2 treatable by parenteral administration of said inhibitory compound.

1 8.   The method of Claim 7 in which the inflammatory disease or condition
2 includes but is not limited to a member selected from the group consisting of acute
3 and chronic infections (bacterial and fungal, including diphtheria and pertussis);
4 acute and chronic serositis (pericarditis, peritonitis, synovitis, pleuritis and
5 tendinitis); uremic pericarditis; acute and chronic cholecystitis; meningitis;
6 encephalitis; arthritis; graft vs. host disease; pernicious anemia; Type I diabetes
7 mellitus; rheumatoid arthritis; multiple sclerosis; transplant rejection; inflammation
8 due to toxic shock or trauma; adult respiratory distress syndrome; reperfusion
9 injury; and inflammatory bowel/Crohn's disease.

ANY REFERENCE TO DRAWINGS
IN THE SPECIFICATION IS
TO BE CONSIDERED NON-EXISTENT
(ART. 14(2))

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/155

US CL :514/634

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/634

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,317,040 (GOLDMAN) 31 May 1994, see the Abstract and column 5, line 5 through column 6, line 21.	1-3, 5, 7, 8
X	US, A, 4,758,583 (CERAMI et al.) 19 July 1988, see column 5, line 44, column 6, lines 32-54 and column 7, lines 34 and 36.	1-8

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 FEBRUARY 1996	27 FEB 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer RAYMOND J. HENLEY III
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